Membrane potential dependent binding of scorpion toxin to action potential Na⁺ ionophore

(Waterichotoxin/neuroblastoma cells/ion transport)

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ABSTRACT Depolarization of neuroblastoma cells causes a 70-fold increase in the apparent dissociation constant K_P for scorpion toxin enhancement of activation of the action potential Na⁺ ionophore by veratridine and a large increase in the rate of reversal of scorpion toxin action. Depolarization also inhibits binding of ¹²⁵I-labeled scorpion toxin to a small number of saturable binding sites on electrically excitable neuroblastoma cells and increases the rate of dissociation of scorpion toxin from these sites. The results suggest that scorpion toxin binds to a regulatory component of the action potential Na⁺ ionophore whose conformation changes on depolarization.

Scorpion venoms have diverse effects on the electrophysiologic properties of axonal and neuromuscular preparations. Exposure of axons under voltage clamp to different species of scorpion venom leads to inhibition of Na⁺ current inactivation, modification of Na⁺ current activation, and suppression of K⁺ current (1-4). Toxic proteins have been purified from five scorpion venoms (5-7). They are basic, have molecular weights of approximately 7000, and lack methionine (5, 6). Treatment of invertebrate giant axons (8) or neuromuscular preparations (9) with high concentrations (0.25-5 μM) of purified scorpion toxins causes electrophysiologic changes similar to whole venom.

Clonal lines of neuroblastoma cells grown in vitro are electrically excitable (10, 11). The Na⁺-dependent portion of the action potential is inhibited by tetrodotoxin at low concentration, suggesting that an action potential Na⁺ ionophore* identical with that in nerve axons is present in these cells (11, 12). Variant cell clones have been obtained which specifically lack the depolarizing phase of the action potential (13, 14). We have previously shown that the neurotoxic alkaloids veratridine, batrachotoxin, and aconitine activate specifically the action potential Na⁺ ionophore of electrically excitable neuroblastoma cells by interaction with a single class of sites (15, 16). Variant clones that lack the depolarizing phase of the action potential do not respond to these toxins, suggesting that they act directly on the Na⁺ ionophore involved in the action potential (15-18). The persistent increase in Na⁺ permeability caused by these drugs can be conveniently measured as an increase in passive ²²Na⁺ uptake into cells. Using this approach we have found that scorpion venom (Leiurus quinquestriatus) acts cooperatively with veratridine, batrachotoxin, and aconitine to activate the action potential Na⁺ ionophore (16). Using this property of the venom as a specific assay, we have purified a toxic protein 80-fold from Leiurus quinquestriatus venom (19). The protein appears homogeneous when analyzed by gel electrophoresis and isoelectric focusing and has a molecular weight of 6700 and an isoelectric point of 9.8 (19). It retains the ability to interact cooperatively with veratridine, batrachotoxin, and aconitine and is effective at very low concentration (apparent dissociation constant K_P = 1-2 nM) (19). In this report we describe the effect of membrane potential on the scorpion toxin enhancement of veratridine activation of the action potential Na⁺ ionophore and on the binding of ¹²⁵I-labeled toxin to electrically excitable neuroblastoma cells. Some aspects of these experiments were reported at the ICN-UCLA Winter Conference on Neurobiology, February, 1976 (17).

EXPERIMENTAL PROCEDURE

Gramicidin A was obtained from Nutritional Biochemicals. N-succinimidyl-3-(3-¹²⁵I)iodo-4-hydroxyphenylpropionate (500 Ci/mmol) was purchased from New England Nuclear. Batrachotoxin was kindly provided by Drs. J. Daly and B. Witkop (Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases). Sources of other chemicals were as described previously (19).

Scorpion toxin was purified approximately 80-fold from the venom of Leiurus quinquestriatus by extraction and ion-exchange chromatography as described previously (19). A 3-(3-¹²⁵I)iodo-4-hydroxyphenylpropionyl derivative of scorpion toxin was prepared by a modification of the procedure of Bolton and Hunter (20). Monoisubstituted toxin was purified by ion-exchange chromatography and shown to have the same biologic activity as native toxin. These procedures and the chemical properties of the derivative will be described in detail in a subsequent manuscript.

Clone N18 of mouse neuroblastoma C1300 was used except where indicated otherwise. Cells were grown essentially as previously described (15, 16). Cultures for experiments were inoculated at 10,000 cells per cm² in multiwell plates and cultured for 6-8 days in Dulbecco’s modified minimal essential medium (DMEM) with 5% fetal bovine serum. ²²Na⁺ uptake was measured as described previously (19). Electrophysiologic recordings were made using an apparatus similar to that described by Nelson et al. (11). Membrane potentials were also calculated from the equilibrium distribution of ³⁵SCN⁻ between the intracellular and extracellular compartments according to the Nernst equation, E_m = 61 mV log ([³⁵SCN⁻]_in/[³⁵SCN⁻]_out).

For measurements of scorpion toxin binding, cells in multiwell plates (1.6 cm diameter) were incubated 30 min at 36° in Na⁺-free medium containing labeled scorpion toxin, the radioactive medium was removed by aspiration, and the cells were washed three times at 36° with 3 ml of wash medium (19) containing 1 mg/ml of bovine serum albumin. In each experiment nonsaturable binding was determined in parallel incubations carried out in the presence of excess (540 nM) unlabeled scorpion toxin. Since the physiologic effect of the toxin is saturable, nonsaturable binding was considered nonspecific and was subtracted from the results.

* The term action potential Na⁺ ionophore refers to the macromolecular structure responsible for the increase in Na⁺ permeability during the depolarizing phase of the action potential in nerve and neuroblastoma cells. The term ionophore is taken to refer in general to ion-transporting molecules without implying a specific mechanism of action.
RESULTS

Dependence of Membrane Potential on K⁺. The membrane potential of excitable cells depends on the permeabilities and concentrations of Na⁺, K⁺, and Cl⁻ approximately as described by the Goldman equation (21). In these experiments we have varied the membrane potential of cultures of neuroblastoma cells by changing the extracellular K⁺ concentration in a Na⁺-free medium in which chloride is the Na⁺ replacement. Membrane potentials measured by microelectrode impalement of visually selected large cells in these cultures are presented in Fig. 1 (O). The average potentials range from −41 mV at 5 mM K⁺ to −0.7 mV at 155 mM K⁺. The slope of the line relating membrane potential to [K⁺] and the absolute value of the potentials are both smaller than expected if K⁺ is the dominant permeant ion. Since the solutions are Na⁺-free, the results suggest that ions other than Na⁺ and K⁺ are significant current carriers in these cells.

Since only the large cells in the population could be successfully impaled under these conditions and it was possible that even these cells were damaged during impalement, we have also measured membrane potentials by an ion distribution procedure. The equilibrium distribution of a permeant ion between the intracellular and extracellular compartments should depend on the membrane potential as predicted by the Nernst equation if the ion is neither pumped nor bound by the cells. This approach to measuring membrane potential has been used in mitochondria (22, 23) and bacteria (24, 25). We have chosen thiocyanate as a permeant ion for these experiments since it is lipid soluble but not amphipathic. The extent of ⁴⁰SCN⁻ up-take at equilibrium changed from 2.8 nmol/mg of cell protein at 5 mM K⁺ to 13.9 nmol/mg of cell protein in 135 mM K⁺, consistent with a membrane potential change of 42 mV. The intracellular volume was 3.3 μl/mg of protein and changed less than 20% as the concentration of K⁺ was changed from 5 to 135 mM. Membrane potentials calculated from these data are illustrated in Fig. 1 (Δ). These potentials represent an average property of the culture population. We consider the agreement between these measurements and those made by microelectrode impalement satisfactory in view of the substantial variability in both sets of data.

Effect of Membrane Potential on Scorpion Toxin Enhancement of Activation of the Action Potential Na⁺ I onophore by Veratridine. Our previous results showed that scorpion venom (16) and purified scorpion toxin (19) act cooperatively with the neurotoxic alkaloids veratridine, batrachotoxin, and aconitine to activate the action potential Na⁺ ionophore. However, unlike the venom, purified scorpion toxin has little ability to activate the action potential Na⁺ ionophore when tested in the absence of an alkaloid toxin (19). The activation by venom must require an additional component removed in purification. Treatment of cells sequentially with scorpion toxin followed by veratridine is as effective as simultaneous treatment with both agents (19). These results suggest that scorpion toxin binds to cells in the absence of alkaloid toxins and remains bound during subsequent treatment with alkaloid toxins and enhances their effect. Thus, in these experiments, we measure the cooperative effect of prior incubation with scorpion toxin on activation of the Na⁺ ionophore by veratridine.

We have studied the effect of membrane potential on the action of scorpion toxin by allowing cells to bind scorpion toxin in solutions of different K⁺ concentration for 20 min, washing away unbound scorpion toxin, and measuring the initial rate of Na⁺ uptake (0.5 min) in medium containing 5 mM K⁺ and a concentration of veratridine (200 μM) that acts virtually instantaneously (15). Depolarization of the cells with K⁺ causes a progressive increase in the apparent dissociation constant (Kₚ) for scorpion toxin from 4.8 ng/ml (0.7 mM) at 5 mM K⁺ to 330 ng/ml (50 mM) at 130 mM K⁺ with little change in the maximum rate of ⁴⁰Na⁺ uptake (Fig. 2). Thus, depolarization of N18 cells reduces their apparent affinity for scorpion toxin.

Similar apparent dissociation constants are obtained if cells are treated with scorpion toxin in medium with varying K⁺
concentrations but the $^{22}\text{Na}^+$ uptake measurements are carried out in 135 mM K+. These results support the conclusion that the membrane potential during treatment with scorpion toxin determines the apparent $K_D$ while the membrane potential during measurements of $^{22}\text{Na}^+$ uptake has little effect.

We have routinely used high extracellular K+ concentrations to depolarize cells in these experiments. In order to test the possibility that the increase in apparent $K_D$ is caused by the changes in K+ concentration per se, we have studied the effect of other depolarizing conditions. Incubation of N18 cells with ouabain for 3 hr in medium containing 130 mM Na+ and 5.4 mM K+ results in complete equilibration of the intracellular Na+ and K+ pools with the medium (18). Incubation of these cells with scorpion toxin in medium containing 130 mM Na+, 5 mM K+, and ouabain results in an apparent $K_D$ of 300–400 ng/ml. However, if a Na+ diffusion potential is established by incubation with scorpion toxin in medium containing 5 mM Na+, no K+, and ouabain, the cells have an apparent $K_D$ for scorpion toxin of 10–20 ng/ml. These results support the conclusion that the membrane potential is the primary determinant of the affinity for scorpion toxin. Other experiments described below in which binding of $^{131}\text{I}$-labeled scorpion toxin has been measured directly provide further support for this conclusion.

The action of scorpion toxin is reversible (19). In order to determine the effect of depolarization on the rate of reversal of scorpion toxin action, N18 cells were incubated with scorpion toxin (107 ng/ml) for 20 min in 5 mM K+ to allow the toxin to act. The cells were then washed free of unbound scorpion toxin and incubated in media with different K+ concentrations. Finally, the initial rate of $^{22}\text{Na}^+$ uptake was measured. The results of these experiments (Fig. 3) show that depolarization of N18 cells causes a large increase in the rate of reversal of scorpion toxin action. The time courses are multiexponential at all membrane potentials studied. The increase in initial slope of depolarization from −44 mV to −4 mV is approximately 15-fold. These data suggest that part of the observed increase in $K_D$ is due to an increase in the rate of dissociation of bound scorpion toxin from its binding site.

**Effect of Membrane Potential on Binding of Scorpion Toxin to N18 Cells.** In order to test the effect of membrane potential on scorpion toxin binding directly, we have studied the binding of a 3-[3-[125]Iodo-4-hydroxyphenyl]propionyl derivative of scorpion toxin (see Experimental Procedure). Since the physiologic effect of the toxin is saturable (Fig. 2) we expect that binding of low concentrations of labeled toxin to its site of physiologic action will be inhibited by concentrations of unlabeled toxin that bind to the same sites and that the observed inhibition will reflect occupancy of the sites by unlabeled toxin. as described by the equation $S^*_\text{bound} = Y/[K_D*][1 + (S/K_D*)]$ for $N \ll S^*$, where $N$ is the concentration of sites, $S$ and $S^*$ the concentrations of unlabeled and labeled toxin, and $K_D$ and $K_D^*$, the dissociation constants of unlabeled and labeled toxin. We observe a component of binding at low concentrations of the labeled toxin that is inhibited by unlabeled toxin at concentrations similar to the apparent dissociation constant for cooperative activation of the Na+ ionophore (Fig. 4, ●) (concentration at 50% saturation $S_{0.5} = 3$ nM, calculated $K_D^* = 1$ nM). This saturable component of scorpion toxin binding is not observed in N103 cells, a neuroblastoma cell line that lacks the depolarizing phase of the action potential (18) and does not respond to veratridine (18) or scorpion toxin (17). Thus, it is likely that this saturable component represents binding of scorpion toxin to the action potential Na+ ionophore.

If a similar binding experiment is carried out in medium containing 135 mM K+, no saturable component of binding is observed (Fig. 4, ○). Thus, depolarization of the cells with K+ inhibits the saturable binding of scorpion toxin. No effect of K+ depolarization on nonsaturable binding is observed.

In medium containing Na+, an increase in Na+ permeability causes depolarization. The antibiotic ionophore gramicidin A forms cation selective ion channels in artificial lipid membranes (26) and depolarizes excitable cells (27). Incubation of N18 cells in Na+ medium (130 mM Na+, 5.4 mM K+) with gramicidin A causes a depolarization from −38 mV to −15 mV and reduces the binding of scorpion toxin 44% (Table 1).

The Na+ permeability of N18 cells can also be increased by activating the action potential Na+ ionophore with veratridine, batrachotoxin, or aconitine. We have shown previously that scorpion venom and scorpion toxin reduce the apparent $K_D$ for activation of the Na+ ionophore by veratridine, batrachotoxin, and aconitine (16, 17, 19), whereas the alkaloids have little effect on the apparent dissociation constant for scorpion toxin (17, 19). As expected from these results, batrachotoxin has no effect on the saturable component of scorpion toxin binding in Na+-free media.
medium containing 5 mM K+. In medium containing 130 mM Na+ and 5.4 mM K+, batrachotoxin depolarizes cells from −38 mV to −5 mV and inhibits scorpion toxin binding 68% (Table 1). The depolarization caused by batrachotoxin is reversed by 1 μM tetrodotoxin, which inhibits the Na+ ionophore non-competitively with respect to batrachotoxin and scorpion toxin (15, 19). Tetrodotoxin also restores scorpion toxin binding. Thus, depolarization of cells by four unrelated agents (elevated K+, ouabain, gramicidin, and batrachotoxin) causes inhibition of scorpion toxin binding.

In order to determine whether the increase in the rate of reversal of scorpion toxin action (Fig. 3) is due to an increase in the rate constant for dissociation of scorpion toxin from its binding site, we have studied the effect of depolarization with K+ on the dissociation of 125I-labeled scorpion toxin from N18 cells. Cells were incubated with a subsaturating concentration of labeled scorpion toxin in 5 mM K+, washed free of toxin, and then incubated in media with various K+ concentrations. The rate of dissociation of the saturable binding component was substantially increased by depolarization (Fig. 5), whereas the rate of dissociation of the nonsaturable component was unaffected by depolarization. As observed in the Na+ uptake experiments (Fig. 3), the time courses are curvilinear and the initial slope is increased at least 15-fold by depolarization. These data confirm that a major effect of depolarization is to increase the rate constant for dissociation of scorpion toxin from its binding site. The curvilinear nature of these data prevents a precise estimate of the values of the rate constant from these data.

The nonlinear time course of dissociation of scorpion toxin probably reflects heterogeneity in the membrane potential of individual cells in the population. Negative cooperativity is not a likely cause for the nonlinear response because concentration-response curves show no sign of cooperativity (Fig. 2), the dissociation rates measured by 32P uptake (Fig. 3) at 100% saturation of the binding sites are similar to those measured by binding of labeled toxin (Fig. 5) at less than 10% saturation, and addition of excess unlabeled toxin does not accelerate dissociation.

**DISCUSSION**

Our results show that depolarization of neuroblastoma cells by four unrelated agents (elevated K+, ouabain, gramicidin, and batrachotoxin) inhibits the binding and action of scorpion toxin. We conclude, therefore, that the observed inhibition is due to the change in membrane potential rather than to a chemical action of the depolarizing agents used.

The data of Fig. 4 and Table 1 show directly that depolarization inhibits scorpion toxin binding. The data of Fig. 2 suggest that the inhibition of scorpion toxin binding is due only to an increase in KD rather than a reduction in the number of scorpion toxin binding sites, since depolarization does not reduce the maximum Na+ permeability attained at high scorpion toxin concentrations. The observed change in KD reflects in large part an increase in the rate of dissociation of scorpion toxin from its binding sites (Figs. 3 and 5).

Our interpretation of these data is that depolarization changes the nature of the scorpion toxin binding site by causing a conformational change which results in an increased rate of dissociation and a decreased affinity for scorpion toxin. We have hypothesized previously that scorpion toxin binds to a regulatory component of the action potential Na+ ionophore (16). Our current results support this hypothesis: (i) We observe saturable binding sites for scorpion toxin in electrically excitable neuroblastoma cells but not in inexcitable cells. (ii) Unlabeled scorpion toxin protects these sites with a KD similar to the apparent KD for scorpion toxin enhancement of veratridine activation of the action potential Na+ ionophore. (iii) Depolarization reduces binding to these sites in parallel with inhibition of activation of the action potential Na+ ionophore by scorpion toxin. We propose, therefore, that scorpion toxin binds to a regulatory component of the action potential Na+ ionophore whose conformation is changed on depolarization.

We have considered two alternative interpretations of our results. (i) It is possible, since scorpion toxin is highly positively charged, that the membrane potential increases the effective concentration of scorpion toxin in an "unstirred" layer along the surface of the cells. In this case, the affinity for scorpion toxin would appear to be greater at more negative membrane potentials because the effective scorpion toxin concentration near the binding sites would be increased. In addition, the effect of depolarization on toxin dissociation could be explained, since toxin in the "unstirred" layer could rebind to sites after disso-

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**Table 1. Inhibition of scorpion toxin binding by depolarization with gramicidin and batrachotoxin**

<table>
<thead>
<tr>
<th>Additions to Na+ medium</th>
<th>Membrane potential (mV)</th>
<th>% Maximum binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>−38</td>
<td>100</td>
</tr>
<tr>
<td>10 μg/ml gramicidin</td>
<td>−15</td>
<td>56</td>
</tr>
<tr>
<td>1 μM batrachotoxin</td>
<td>−5</td>
<td>32</td>
</tr>
<tr>
<td>1 μM batrachotoxin</td>
<td>−41</td>
<td>100</td>
</tr>
</tbody>
</table>

N18 cells were incubated for 30 min at 36° in medium with 130 mM Na+, 0.3 nM 125I-labeled scorpion toxin, the additional components indicated in the table, and increasing concentrations of unlabeled scorpion toxin as in Fig. 4. Bound toxin was measured as described in *Experimental Procedure*. Saturable binding was determined from a computed least-squares fit of the data. Membrane potentials were measured using the 32P distribution technique described in Fig. 1.

**FIG. 5. Effect of K+ on rate of dissociation of 125I-labeled scorpion toxin.** N18 cells were incubated for 20 min at 36° with 0.3 nM 125I-labeled scorpion toxin, washed to remove unbound toxin, and incubated again for the indicated times in Na+ free medium containing 5 mM (●), 10 mM (△), 25 mM (□), 60 mM (▲), or 135 mM K+ (●) and choline Cl such that [K+] + [choline+] = 130 mM. An identical set of cultures was incubated with labeled toxin in the presence of excess unlabeled toxin (555 ng/ml). Nonsaturable binding to this set of cultures was subtracted from the experimental data.

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cation, reducing the observed dissociation rate. While we cannot directly exclude an effect of this type on the binding constant, we can exclude an effect on dissociation rate since, in the experiment of Fig. 3, addition of a large excess of unlabeled toxin, which would also be concentrated in the hypothetical "unstirred" layer and prevent rebinding of labeled toxin, did not increase the dissociation rate in 5 mM K+.

(4) It is possible that the membrane potential has a direct electric field effect on the binding of the positively charged toxin to a negatively charged site on the membrane. This mechanism is energetically unfavorable. It is probable that the electric field due to the membrane potential extends only to the surface of the high resistance hydrocarbon portion of the membrane. For the large, highly charged toxin to enter this field, it must enter the hydrocarbon portion of the membrane or penetrate the transmembrane ion channel. While neither possibility seems likely, this mechanism cannot be excluded.

Assuming that our interpretation is correct, it is of interest to compare the membrane potential dependence of scorpion toxin binding with the membrane potential dependence of m and h parameters which describe the processes of activation and inactivation of the Na+ ionophore in the model of Hodgkin and Huxley (28). The apparent Kp increases e-fold in 9.6 mV (Fig. 1). Plots of log m or log h versus membrane potential derived from the data of Hodgkin and Huxley on squid giant axon (28) have similar shape with an e-fold increase 11.6 mV or 9.8 mV for m and h, respectively. However, the curves for the change of m and h parameters with membrane potential are shifted to more negative potentials, approximately 20 and 40 mV, respectively.

Our initial studies of binding at saturating toxin concentrations suggest an approximate value of 10 ± 5 fmol of binding sites per mg of cell protein or five sites per μm² of surface membrane. The density of binding sites for tetrodotoxin in unmyelinated nerves is similar (29–34). Comparison with ion flux data (19) leads to an estimate of 2 × 10⁶ ions/min per site or 5 pmho/per site (5 pS/site) for the ion transport capacity of the Na+ ionophore. Similar values have been derived for squid axon from tetrodotoxin binding data (35, 36) or noise analysis (37).

Our results suggest that scorpion toxin binds specifically to a membrane potential dependent regulatory component (gate) of the Na+ ionophore. Tetrodotoxin and saxitoxin appear to bind to a separate site (15, 16), which coordinates monovalent cations during transport (38–40). Thus, toxins provide specific radioactive ligands for two separate functional components of the action potential Na+ ionophore. The stoichiometry and physical relationship between these two components remain to be determined.

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